Review Article
Cancer stem cells: a major culprit of intra-tumor heterogeneity

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Abstract: Cancer is recognized as a preeminent factor of the world's mortality. Although various modalities have been designed to cure this life-threatening ailment, a significant impediment in the effective output of cancer treatment is heterogeneity. Cancer is characterized as a heterogeneous health disorder that comprises a distinct group of transformed cells to assist anomalous proliferation of affected cells. Cancer stem cells (CSCs) are a leading cause of cancer heterogeneity that is continually transformed by cellular extrinsic and intrinsic factors. They intensify neoplastic cells aggressiveness by strengthening their dissemination, relapse and therapy resistance. Considering this viewpoint, in this review article we have discussed some intrinsic (transcription factors, cell signaling pathways, genetic alterations, epigenetic modifications, non-coding RNAs (ncRNAs) and epitranscriptomics) and extrinsic factors (tumor microenvironment (TME)) that contribute to CSC heterogeneity and plasticity, which may help scientists to meddle these processes and eventually improve cancer research and management. Besides, the potential role of CSCs heterogeneity in establishing metastasis and therapy resistance has been articulated which signifies the importance of developing novel anticancer therapies to target CSCs along with targeting bulk tumor mass to achieve an effective output.

Keywords: Cancer stem cells, tumor heterogeneity, metastasis, therapy resistance

Introduction

With the advancement of cancer genetics, it is evident that cancer is a heterogeneous ailment that involves the acquisition of genetic and epigenetic modifications to assist cancerous cell proliferation, dissemination and therapy resistance [1]. Cancer heterogeneity has been categorized into two distinct types: intertumor heterogeneity and intratumor heterogeneity. Intertumor heterogeneity involves variations in the tumor of the same tissue in different individuals or variations in the tumor of different tissues in the same individual. On the other hand, intratumor heterogeneity encompasses variability within a single tumor. Intertumor heterogeneity lays the foundation of tumor classification in different types and subtypes on the basis of histological appearance, specific expression markers, and divergent genetic profiles. Where-as, intratumor heterogeneity entangles tumor efficacious treatment and complete eradication [2].

CSCs are oncogenic in nature and imputed as key drivers of tumor heterogeneity [3]. Although at first it was presumed that CSCs are a homogenous population of cells, with time it has been proved that CSCs are phenotypically and functionally diverse. There is ample evidence that CSCs hierarchy leads to the intratumor heterogeneity [4-6]. In a contemporary study, Tabuchi and coworkers have unmasked CSCs functional heterogeneity at clonal level. They showed that CSCs in uterine endometrial carcinoma comprised two distinct sub-clones i.e. highly tumorigenic clone and treatment-refractory clone [7]. Moreover, recent literature also represents that CSCs are not static but dynamic populations of cells that are continually transformed by the cells' extrinsic and intrinsic factors [8].

Bonnet and Dick were the first researchers to disclose CSCs in human acute myeloid leukemia (AML). In accordance with their pioneer work, just like healthy hematopoietic stem cells (HSCs) leukemia stem cells (LSCs) bear CD34$^+$CD38$^-$ phenotype [9]. Recent investigations with large sample sizes display that LSCs are not only restricted to CD34$^+$CD38$^-$ fraction but CD34$^+$CD38$^+$ fraction also harbors leukemogenic cells [10, 11]. Confirming LSCs heterogeneity Taussig and collaborators stated that LSCs in NPM1-mutation harbor CD34$^-$ phenotype while CD34$^+$ phenotype is only associated with NPM1-mutated AML that additionally possess higher risk to develop FLT3^{IDT} mutation [12]. Results from some other groups also made explicit that both CD34$^-$ and CD34$^+$ phenotypes can exist in NPM1-mutated AML [13].

Like leukemia, solid tumors also harbor several different subpopulations of stem-like cells. In the year 2003, Al-Hajj and his companions first identified breast cancer stem cells (BCSCs) with CD44$^+$CD24$^{-/L}$LIN$^-$ pattern [14]. Later, another group detected an elevated level of ALDH1 in both normal and CSCs. However, cells bearing both CD44$^+$CD24$^{-/L}$ and ALDH$^+$ markers were more malignant than the cells bearing a single marker only [15]. Further describing combination markers for BCSCs enrichment Pece and coworkers revealed that cells with CD24^{H}CD49^{H}DNER^H, CD24^{H}CD49^{H}DLL1^H and CD49F^{H}DLL^{H}DNER^H phenotypes could be utilized as markers for BCSCs identification [16]. On the other hand, to reveal BCSCs heterogeneity, Hwang-Verslues and coworkers reported a highly tumorigenic subpopulation of BCSCs with PROCR$^+$/ESA$^+$ phenotype instead of CD44$^+$CD24$^{-/L}$ and ALDH$^+$ signature [17]. Similarly, in ER-α-negative breast cancer, not only CD44$^+$CD24$^-$ and CD44$^+$CD24$^+$ cell populations were found oncogenic but they also found a third more malignant phenotype CD44$^+$CD49f^{H}CD133/2^H of BCSCs [18]. Furthermore, heterogeneous markers have been described for enrichment of CSCs in the tumors of other organs [19]. For example in glioblastoma, CD133, CD44, CD15, A2B5, integrin α6 and LICAM markers have been reported for CSCs identification [20, 21]. Cell surface markers, in particular CD133, CD44, ALDH, CD166, CD133$^+$ESA$^+$, CD166$^+$CD44$^+$, and CD166$^+$EpCAM$^+$ are exploited for lung cancer stem cells (LCSCs) isolation, whereas for bladder cancer stem cells detection CD44, CD67LR, ALDH1A1, BCMab1 and EMA markers have been described in the literature [22].

In light of these data, it can be stated that CSCs have been found to be highly heterogeneous in different malignancies, and only one type of marker is not enough to isolate or target a particular CSCs from the bulk of the tumor mass. Hence, this review article is intended to summarize recent findings of potential mechanisms of CSCs heterogeneity that how these are equipped with stemness features. At first, we have magnified the intrinsic factors, like transcription factors, signaling pathways, genetic alterations, epigenetic modifications, ncRNAs and epitranscriptomics that we regard as the inherent properties of self-renewal. Secondly, we have pinpointed the role of TME as an extrinsic factor that can significantly alter phenotype of these cells. Additionally, the function of this heterogeneity in cancer dissemination and therapeutic resistance has been documented, along with discussing some already established therapeutic approaches to target CSCs.

Models of CSCs heterogeneity

Historically, two contrasting models were proposed to describe cancer heterogeneity. These models were named: the clonal evolution (CE) model and the cancer stem cell (CSC) model. The CE model was emanated from Darwin's evolution theory, where a single cell is responsible for tumor initiation. This model states that with time stochastic changes occur within a cell and as a consequence of cellular division these changes can pass to the daughter cells. The daughter cells are prone to acquire more and more perturbations and as a result of natural selection, only the most adopted clone will survive while the clones with less adaptation will extinct out eventually (**Figure 1**) [23]. On the contrary, the CSC model explicates that cancerous cells harbor a small subgroup of stem cells with cancer-initiating potential. These cells are recognized as "cancer stem cells (CSCs)" and just like healthy stem cells, CSCs hold self-renewable and differentiation capabilities. This model intimates that due to the stem cells differentiation cancerous cells proliferate in a hierarchical fashion and thus, only CSCs are accountable for tumor induction, propagation, infiltration and relapse (**Figure 1**) [24].
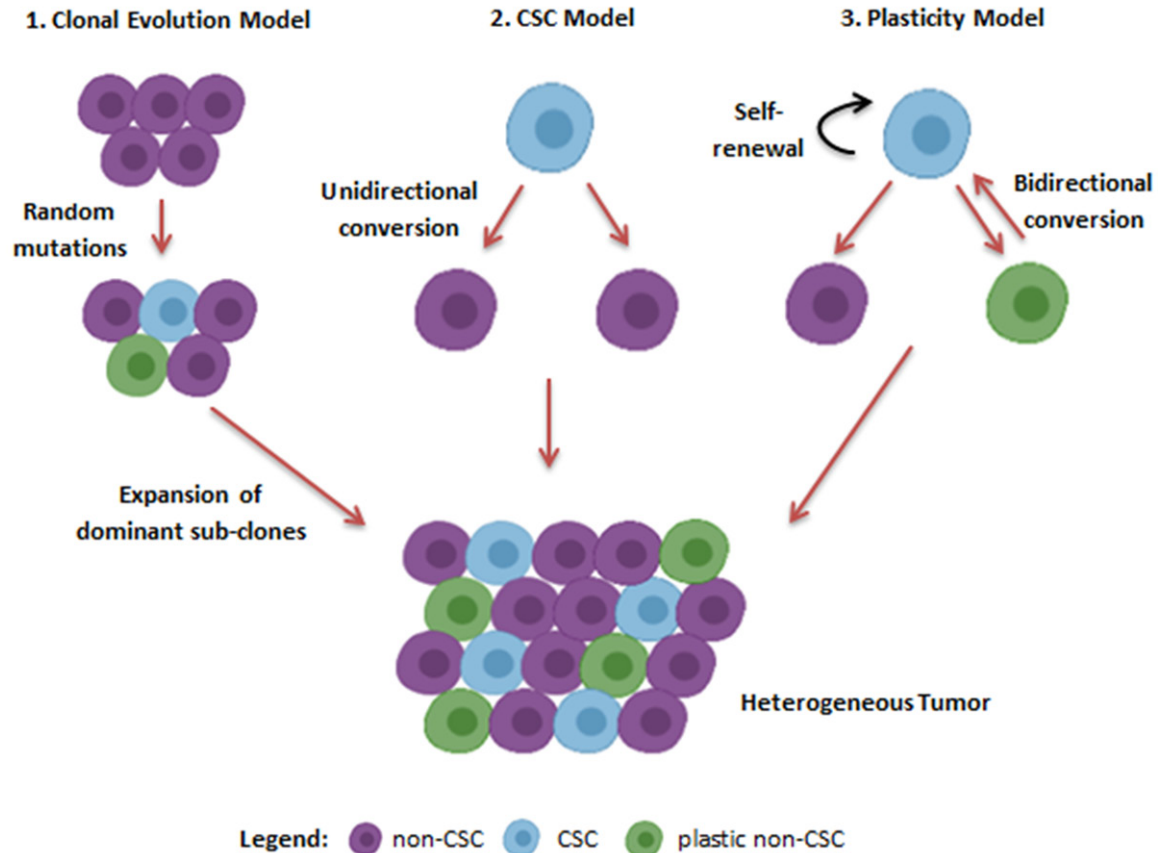
Figure 1. Models of Cancer heterogeneity. According to the clonal evolution model stochastic mutations give raise to the divergent sub-clones which finally make the tumor heterogeneous. The second model or the CSCs model explicates that small population of stem cell like cells within cancer initiates and maintains cancer heterogeneity as they possess the ability to differentiate into any cell type. This model represents that the conversion of CSCs to non-CSCs is irreversible process. On the contrary, according to the plasticity model this conversion is bidirectional. Hence, cancer cells change their phenotype in response to the dynamic microenvironment.

Although in the beginning, it was believed that these models are not mutually exclusive as some cancers follow the CE model, while others follow the CSC model. But this concept was not enough to describe cancer heterogeneity completely. For example, in chronic myeloid leukemia (CML), a cancer that is completely directed by CSCs, imatinib treatment caused clonal evolution [25]. Similarly, phenotypic plasticity has been observed in LSCs upon chemotherapy treatment [26]. These observations led to a third evolving model called the plasticity model, in which CSCs and non-CSCs harbor the ability to interchange their states among each other (**Figure 1**). Thus, the CE model and CSCs model are not mutually exclusive and the plasticity model further facilitates complicacy to the well-explained paradigm of cancer heterogeneity.

**Factors contributing to the CSCs heterogeneity**

In order to understand CSCs heterogeneity, it is essential to comprehend how stemness attributes are set up in cancerous cells. Therefore, in this section different intrinsic and extrinsic elements have been discussed which contribute to CSCs stemness initiation and maintenance (**Figure 2**).

Intrinsic factors

Transcription factors

OCT4, SOX2, KLF4, c-MYC and NANOG are recognized as master pluripotency factors as they can reprogram somatic cells into induced pluripotent stem cells by suppressing differentiation-associated genes or upregulating pluripo-
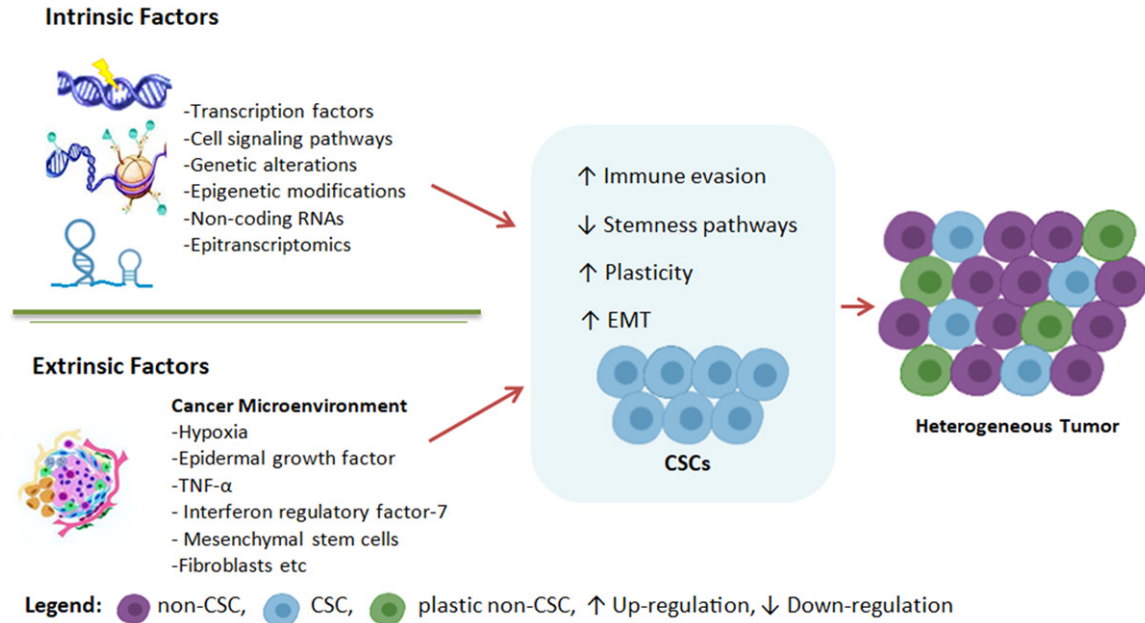
Figure 2. Factors regulating CSCs to promote cancer heterogeneity. Cellular intrinsic and extrinsic factors up-regulate stemness traits in cancerous cells. They also help CSCs to evade immune response and change their phenotype to promote cancer.

tency and self-renewal related genes. Accumulating evidence shows increased expression of these master regulators in CSCs [27]. For instance, increased expression of OCT4 has been found to be positively linked with chemoresistance and inversely linked with clinical prognosis in the majority of the tumors [28]. Its increased expression also shows an association with glioma grades [29]. Likewise, SOX2 promotes metastasis and cancer stem-cell function in invasive cutaneous squamous cell carcinoma [30]. Gangemi and companions represented that SOX2 is essential for sustaining tumorigenesis and self-renewal of glioblastoma-initiating cells (GICs) as its knockdown halted glioblastoma tumorigenicity and proliferation [31]. KLF4, which is another important transcription factor of pluripotency, has been found to perform a dual role in cancer maintenance. Sometimes, it acts as an oncogene as in the melanoma xenograft model, its depletion causes inhibition of cancer [32]. In the same fashion, its overexpression in osteosarcoma cells caused the formation of osteosarcoma cancer stem cells with chemoresistance and metastasis potential [33]. On the contrary, in some other cancers, its anticancer effect has also been documented as in liver cancer, bladder cancer, non-small-cell lung carcinoma

and leukemia its downregulation has been observed [27]. The three different isoforms of c-MYC are also overexpressed in different cancers to maintain CSCs characteristics [27]. Similarly, NR5A2 has been found to increase NANOG expression to maintain stemness of lung cancer [34]. Moreover, these factors also work in a combination of other transcription factors i.e. STAT3, ZIC3 and HESX1 to promote CSCs tumorigenic potential, transformation and metastasis [35].

Besides maintaining stemness properties of CSCs, transcription factors also play a key role in promoting tumor heterogeneity and plasticity. For example, in glioblastoma, a core set of four transcription factors (SOX2, OLIG2, POU3F2 and SALL2) can reprogram differentiated glioblastoma cells into tumor-initiating glioblastoma stem-like cells [36]. A recent study showed that increased c-MYC expression has been found to be linked with intratumor heterogeneity in triple-negative breast cancer cells [37]. Similarly, the transcription factor YB-1 was found to be responsible for the reversion of differentiated tumor cells into CSCs [35]. Hence, it is evident that transcription factors play a dominant role in maintaining tumor heterogeneity by regulating CSCs proliferation and differentiation.

Cell signaling pathways

Many cell signaling pathways that control normal stem cells homeostasis are found to be highly activated or repressed in cancerous cells. Thus, leading to the activation and proliferation of CSCs [27]. These pathways are not independent of each other but form a complicated signaling network to regulate and propagate tumors [38]. Different endogenous or exogenous genes and ncRNAs are implicated in the regulation of these signaling pathways. In addition, these can also stimulate the expression of some downstream genes associated with the apoptosis, proliferation and metastasis of CSCs [38]. In this section, some key signaling pathways are discussed that support CSCs formation and function.

Notch signaling pathway: In mammals, the Notch signaling pathway has four receptors: (Notch1, Notch2, Notch3, and Notch4) and five ligands: (DLL1, DLL3, DLL4, JAG1 and JAG2) [39]. These ligands and receptors are transmembrane proteins that participate in cellular communication [27]. This pathway gets activated when a receptor binds to a ligand on an adjacent cell in a juxtacrine manner, resulting in the translocation of the Notch intracellular domain to the nucleus to activate transcription of targeted genes [40].

The Notch pathway has been found abnormally activated in different malignancies. However, its exact role in the maintenance of CSCs has just been recently understood. It is because different cancers express different Notch receptors and ligands [41]. Hence, it functions as either tumor promoter or suppressor, depending upon the type of cancer involved. As a tumor promoter, Notch signaling is overactivated in pancreatic, gastric, colon and breast cancer cells [42-44]. While this pathway is found suppressed in liver, prostate, lung and skin cancer cells [45-49]. This dual nature of the Notch signaling pathway is controlled by the microenvironment [50]. Studies conducted on the role of Notch pathway in CSCs revealed that its activation promotes the development and differentiation of CSCs. For example, in hepatocellular carcinoma (HCC) and breast cancer, aberrant activation of the Notch1 and Notch4 enhanced metastasis and self-renewal properties of CSCs [51, 52]. Likewise, patient-derived

pancreatic cancer stem cells (PCSCs) exhibited increased expression of Notch1, Notch3, JAG1, JAG2 and HES1, suggesting the potential role of Notch signaling in CSCs maintenance. Moreover, overexpression of DLL4 in gastric cancer cells resulted in increased self-renewal and metastasis of gastric cancer stem cells (GCSCs) [53]. In glioblastoma, DLL1 requires actin cytoskeleton regulator ARP2/3 complex to activate Notch signaling for maintaining stem cells phenotype [54]. Notch pathway activation is also required for maintaining the undifferentiated state of CSCs, as in adenomas its inhibition caused differentiation of adenoma cells into goblet cells [55]. Furthermore, some endogenous genes also activate the Notch pathway. In cervical cancer, MAP17 sequestrates NUMB to differentiate tumor cells into CSCs [56]. Finally, it has also been observed that different tumorigenic factors also maintain CSCs phenotype by activating Notch. In osteosqamous cell carcinoma cells, TNF-α mediated Notch activation promotes CSCs phenotype [57]. Similarly, hypoxia-induced JAG2 activation enhances the invasiveness properties of lung and BCSCs [58, 59]. Additionally, BMP-4 activates the Notch pathway to promote epithelial-to-mesenchymal transition (EMT), a process that is cardinal to maintain cancer cells' plasticity and stem cell properties in breast cancer cells [60]. Collectively, these studies clearly express that the Notch pathway is essential for the survival and growth of CSCs in different cancers.

Wnt signaling pathway: The Wnt pathway is a highly intricate signaling pathway that plays a crucial role in the proliferation and maintenance of CSCs [61]. This pathway has been divided into three subtypes: the canonical Wnt pathway, the non-canonical planar-cell polarity pathway and the non-canonical Wnt-calcium pathway [62]. Among these, the canonical Wnt pathway is the most understood and its inhibition has remained a topic of interest in cancer biology. In general, it incorporates 19 ligands and around 15 receptors [63]. The activation of the Wnt pathway has been found different in different malignancies. In some cases, it gets activated by mutations, like FLT3[IDT] mutation in AML [64], APC mutation in colorectalcarcinoma [65], AXIN mutation in gastric cancer [66] and β-catenin mutation in liver cancer [67]. While in other cases, growth factors from the TME, like

HGF, PDGF, VEGF and PGE2 activate this pathway [68]. Tumor dormancy is a critical phenomenon that ultimately develops cancer metastasis and recurrence. Studies suggest that activated Wnt signaling transforms dormant CSCs into active CSCs to promote tumorigenesis [69]. Moreover, activated Wnt signaling has also been found to be associated with the self-renewal of CSCs. Different proto-oncogenes promote this process by activating the Wnt pathway [70]. For instance, in colorectal cancer, EZH2 enhances colorectal cancer stem cells (CCSCs) expansion by activating Wnt/β-catenin signaling [71]. Whereas in prostate cancer, TERT (a telomerase reverse transcriptase) activates downstream targets of Wnt via making a complex with β-catenin [72]. PKM2 increases the proliferation of BCSCs by activating the Wnt pathway [73]. In the same manner, capillary morphogenesis gene 2 upregulates β-catenin expression to regulate GCSCs phenotype [74].

Aberrant Wnt activation also regulates the dedifferentiation of CSCs. PMP22, which normally causes differentiation of CSCs, was found depleted after Wnt activation [75]. TRAP1 halted the differentiation of CSCs via regulating phosphorylation of the β-catenin [76]. Furthermore, in esophageal squamouscell carcinoma, LGR5 caused Wnt activation to restrain CSCs differentiation [77]. Studies also show the role of Wnt signaling in inhibiting apoptosis of CSCs. DACT1 antagonizes the Wnt/β-catenin pathway to increase apoptosis in BCSCs [78]. Likewise, DKK2-mediated downregulation of β-catenin in BCSCs leads to the Go/G1 phase arrest and apoptosis in breast cancer [79]. Wnt signaling also promotes metastasis of CSCs. In the human colon, pancreatic and lung CSCs upregulation of CD44v6 causes Wnt activation which leads to cancer metastasis and invasion [80-82]. Besides, CDH11 was found to decrease the migration ability of CCSCs by deactivating Wnt [83]. These studies suggest that aberrant Wnt activation plays a significant role in maintaining CSCs' dedifferentiation, apoptosis inhibition, and invasion.

Hedgehog (HH) signaling pathway: The HH signaling pathway is another complex regulatory network, that promotes tumorigenesis of different organs by regulating CSCs [27]. This pathway is comprised of three extracellular ligands: Sonic hedgehog, Desert hedgehog and Indian hedgehog, PTCH receptor, Smoothened protein (SMO) and three GLI transcription factors: GLI1, GLI2 and GLI3 [27]. Studies show increased expression of HH pathway mediators in different CSCs. In human multiple myeloma-derived progenitor cells, high expression of SMO gene and increased transcriptional activity of GLI1 were observed [84]. In the same manner, human gliomas showed increased expression of HH signaling-associated genes PTCH1, GLI1 and SHH [85]. Upon treatment with HH pathway inhibitor, enhanced expression of master pluripotency factors (SOX2, OCT4 and NANOG) was observed, which caused inhibition of self-renewal and proliferation abilities of CSCs [85]. Furthermore, in the SMO-deficient murine CML model, ectopic expression of the SMO gene enhanced CML progression by increasing the frequency of CSCs [86].

Different oncogenes and tumor suppressor genes also regulate tumorigenesis by modulating HH signaling in CSCs. In medulloblastoma stem cells, BCL6 caused repression of the HH pathway by inhibiting GLI1 and GLI2 [87]. In glioma, SCUBE2 inhibited the HH pathway to halt the proliferation and migration of glioma cancer stem cells [88]. Also, in PCSCs, vasohibin 2 caused the deactivation of SMO, GLI1 and GLI2 [89]. HH signaling also promotes CSCs-mediated tumor metastasis. CSCs isolated from human colon cancer liver metastasis patients exhibited increased expression of GLI1, GLI2 and HIP genes. Besides, these CSCs also expressed a high level of SNAIL 1 (an EMT-associated gene), as compared to nonmetastatic controls [90]. In addition, RUNX3 a tumor suppressor transcription factor inhibited metastasis and stemness properties of CCSCs by causing GLI1 intracellular ubiquitination [91]. These studies indicate that overactivated HH signaling assists CSCs growth, proliferation and metastasis.

JAK/STAT signaling pathway: The JAK/STAT signaling cascade is one of the simplest signaling pathways that gets activated by different ligands, like cytokines, hormones and growth factors [92]. This pathway regulates different important biological processes, such as self-renewal, neurogenesis and hematopoiesis in embryonic stem cells (ESCs) [93]. Several lines of evidence indicate aberrant JAK/STAT activation in different CSCs. For example, overactivation of the JAK/STAT pathway-associated

genes, such as STAT1, IFNK, IFNGR, CSF2 and IL-6 has been observed in prostate cancer stem cells (PrCSCs) [94]. Whereas the activated form of STAT3 was found to be upregulated in BCSCs [42]. Overactivation of JAK/STAT signaling also promotes stemness properties in human glioblastoma. As in an analysis of glioblastoma, patient-derived GICs showed TGF-β and LIF-mediated JAK/STAT pathway activation that led to the increased self-renewal and reduced differentiation capabilities in GICs [95]. In endometrial cancer, IL-6 caused activation of the JAK1/STAT3 pathway in ALDH^H and CD126$^+$ cells [96]. Furthermore, in ovarian cancer, OCT4 promoted activation of the JAK2/STAT3 pathway in CSCs [97]. The JAK/STAT pathway also plays an important role in the regulation of LSCs [98]. Constitutive JAK/STAT activation has been observed in the LSCs isolated from AML patients. Upon treatment with JAK1/2 inhibitor, LSCs lost their tumorigenic ability as in secondary transplant they failed to develop leukemia in immunocompromised mice [99]. The JAK/STAT signaling also participates in CSCs-mediated therapy resistance. As in colorectal cancer, the JAK2/STAT3/CCND2 axis promoted CSCs growth after radiotherapy by limiting apoptosis and accelerating clonogenicity [100]. Similarly, in myxoid liposarcoma cancer stem cells, JAK/STAT signaling activation led to chemotherapy resistance [101]. Thus, the JAK/STAT pathway serves an important role in promoting stemness properties and CSCs-mediated therapy resistance.

NF-κβ signaling pathway: NF-κβ is another complex and essential signaling cascade that plays a crucial role in regulating inflammatory and immune responses [102]. Aberrant NF-κβ has been observed in hematological malignancies, breast tumors, head and neck squamous cell carcinoma (HNSCC), gastrointestinal, gynecological and genitourinary cancers [27]. Although previously its role was unclear in the regulation of CSCs, recent studies have shed light on its essential role in CSCs functioning, maintenance, proliferation and metastasis. In ovarian cancer, CD44$^+$ cells showed increased expression of different NF-κβ pathway-associated genes to promote tumorigenesis [103]. In another study, BCSCs displayed increased level of NF-κβ-inducing kinase to support the proliferation and metastasis of BCSCs [104]. IKKβ upregulated LIN28 expression to promote BCSCs lung metastasis [105]. Moreover, in a recent study, Nancy and colleagues have also identified the role of NF-κβ signaling cascade in maintaining BCSCs plasticity to promote its aggressiveness and invasion [106]. Similarly, in the colorectal cancer mice model, PGE2-mediated NF-κβ activation promoted CSCs expansion and metastasis [107]. Finally, CCR7 in association with its ligand CCL21 caused NF-κβ activation in CD133$^+$ CCSCs to enhance their survival, while inhibiting apoptosis [108]. These investigations demonstrate that the activated NF-κβ pathway regulates CSCs' properties to increase tumor metastasis and aggressiveness.

PI3/AKT/mTOR signaling pathway: PI3/AKT/mTOR is an important intracellular signaling pathway that regulates the cell cycle. This pathway has been studied extensively for its role in developing cancer. Moreover, its function in driving therapy resistance in various tumors has also been reported. However, limited data is available regarding its role in stemness maintenance [109]. PI3/AKT/mTOR signaling cascade accelerates metastasis and invasion of PCSCs and PrCSCs [110, 111]. Deactivation of PTEN, a tumor suppressor gene, induced PI3K activation in CD133$^+$/CD44$^+$ PrCSCs to stimulate their survival and tumorigenesis [112]. In HNSCC, aberrant PI3K signaling improved the proliferation and metastasis capabilities of ALDH$^+$CD44^H CSCs [113]. Overactivation of mTOR has also promoted survival and self-renewal in nasopharyngeal cancer stem cells and BCSCs [114, 115]. In HCC, mTORC2 increased the expression of EpCAM [116]. Whereas, in CCSCs, activation of mTORC1 increased ALDH1 activity [117]. In another analysis, activated PI3K/AKT pathway promoted SOX2 expression in CCSCs to induce the production of radiotherapy resistant CSCs [118]. Therefore, it is evident that the PI3K/AKT pathway promotes CSCs-mediated therapy resistance, metastasis and invasion.

The aforementioned CSCs regulatory pathways are not always linear, as in some cases crosstalk among different signaling cascades also occur to regulate the properties of tumor-initiating cells. HH and PI3K pathways work together to regulate biliary tract CSCs and PrCSCs [119, 120]. Likewise, the association between NF-κβ and Wnt/β-catenin pathway occurs in colorectal cancer to support self-renewal and proliferation of CSCs. In this case, β-catenin increased

TNFRSF19 expression to overactivate, NF-κβ pathway [121]. However, in some cases, negative association also occurs between these regulatory pathways. For example in colon, breast and liver cancer, NF-κβ activation caused deactivation of β-catenin/TCF activity by increasing tumor suppressor LZTS2 [122-124]. Cross-talk of CSCs regulatory pathways also participate in establishing therapy resistance in tumor cells. For instance, PI3K and Notch pathways work in association to drive PI3K therapy resistance in triple-negative breast cancer [125]. In HH activated medulloblastoma a mice model, activation of PI3K was observed to sustain therapy-resistant CSCs in the perivascular niche [126]. Moreover, the association of these signaling pathways also regulates CSCs to promote tumor growth and metastasis. IL-6/JAK/STAT3 and TGF-β/Smad signaling work together to promote LCSCs metastasis [127]. The combination of Notch, IKK/NF-κβ and some other pathways enhanced the proliferation and migration of CD133+ skin cancer stem cells [128]. Finally, the binding of IL-17E to IL-17B has been observed in HCC to activate NF-κβ and JAK/STAT3 pathways for regulating CSCs growth [129].

Genetic alterations

In the journey of cell division, cells agglomerate various genomic alterations just by chance. In an exquisite study, Tomasetti and Vogelstein revealed a strong correlation between no of stem cell division and tissue-associated cancer risk. This work indicates that stem cells are the main players of arising cancer in humans [130]. Although neoplastic transformation can eventuate in any cell type i.e. stem cells, progenitor cells, or differentiated cells, the stem cells are preferentially dominant targets for malignant transformation [131]. Thus, CSCs utilize normal molecular mechanisms of healthy stem cells, like self-renewal and proliferation to initiate and propagate cancer [24]. This phenomenon can be observed well in hematological malignancies in which the acquisition of different forms of chromosomal translocations in HSCs produce different types of leukemia. For example, BCR-ABL translocation in these progenitor cells originates CML [132], MLL-ENL translocation in CD34+ hematopitic stem cells and progenitor cells leads to B-lineage and monocytic leukemia [133] and AML1-ETO in HSCs leads to AML [134]. It is well known that TET2 dele-

tion in the hematopoietic region is linked with CMML/MPN, while FLT3^{IDT} mutation in TET2 deprived hematopoietic stem cells transforms them to AML [135]. Furthermore, these two germline perturbations synergistically exert a pathogenic impact on the bone marrow niche by promoting leukemogenesis [135]. These types of genetic mutations have also been observed in the initiation of solid tumors as *PTEN* loss in human neural stem cells results in the formation of glioblastoma stem cells (GBSCs) [136]. Lee and coworkers have provided direct molecular genetic proof that neural stem cells that reside in the brain's subventricular zone carry driver mutations to originate glioblastoma [137]. Similarly, *SMAD4* and *PTEN* mutated LRG5+ stem cells originate invasive intestinal-type gastric cancer in mice [138].

Oncogenic transformations have also been reported to be responsible for promoting the aggressiveness of cancer [139]. In a recent study, Weng and colleagues showed that Kras^{G12D}/p53 loss caused upregulation of cancer stem cell-like traits (CD24, CD133 and EpCAM) in the prostate cancer mice model, which enhanced tumorigenic potential and bone metastasis of prostate cancer [140]. Moreover, oncogenic transformation in stem cells can also produce immune surveillance response in tumor cells. For instance, neoplastic alterations in human mesenchymal stem cells reduced their immunogenicity and increased their ability to inhibit mitogen-driven T-cells proliferation [141]. In the same manner, mutated stem cells are found to express an elevated level of immune modulators such as CTLA4 and CD274 (PD-L1) [142]. A current study confirms this phenomenon by revealing that PARP upregulation in LSCs inhibited NK-G2D-L expression which led to evade immune response [143].

Just like a healthy stem cell, normal differentiated cells also depict a permissive pool for CSCs generation [144]. The two most prevalent brain tumors i.e. neuroblastoma and glioblastoma are presumed to be initiated from the dedifferentiation of mature neurons and glial cells [145]. Köhler and colleagues have provided *in vivo* evidence that differentiated, mature pigment-producing melanocytes cells are cells of origin of melanoma. Using the lineage tracing approach they showed that pigment-producing melanocytes dedifferentiate and ex-

pand to originate BRAF^{V600E} induced melanoma [146]. Similarly, the oncogenic isoform of C/EBPα dedifferentiates hepatocytes into CSCs to initiate HCC and aggressive hepatoblastoma [147].

Epigenetic modifications

Even though genetic perturbations play a major role in tumorigenesis, these changes alone are not enough to establish all hallmarks of cancer. Therefore, genetic and epigenetic alterations go hand in hand to maintain and propagate tumors. For instance, loss of APC in LRG5$^+$ intestinal CSCs led to DNA methylation to further increase CSCs signature [148]. Additionally, Arg-882-mutated *DNMT3a* in combination with *RAS*-mutation induced LSCs to originate AML [149]. Likewise, mutated DNMT3a induced CpG hypermethylation and histone acetylation to deregulate stemness pathways [149]. Furthermore, irregular DNA methylation has been observed frequently in different malignancies [150, 151]. In Aldeflour$^+$ BCSCs, DNA hypermethylation of *TAP* gene helps to evade T cells [152].

Epigenetic alterations also regulate the plasticity of CSCs. As the existence of functionally distinctive cellular subtypes i.e. CSCs and non-CSCs in tumor leads to heterogeneity, epigenetic modifications can effectively switch CSCs into non-CSCs in established cancers. An example of this mechanism can be observed in liver cancer, in which modulation of Nanog promoter methylation results in the switch between CSCs and non-CSCs [153]. Emerging evidence suggests that epigenetic modifiers contribute significantly to sustain and support oncogenic aptitude of CSCs. As ZSCAN4 mediated NANOG and OCT3/4 promoter hyperacetylation leads to enhanced CSCs features in HNSCC [154]. Collectively, in light of the mentioned literature, it's evident that genetic and epigenetic modifications are mandatory factors for installing and maintaining stemness traits.

Non-coding RNAs

Although, in the beginning, there was consensus among the scientific society that only a minority of the human genome (less than 2%) encodes for protein while the remaining major portion (98%) was considered junk. With the advancement of scientific research, RNA-se-

quencing technology has revealed the importance of this junk portion in the regulation of gene expression [155]. Non-coding RNAs in particular, long non-coding RNAs (lncRNAs) and microRNAs (miRNAs) perform a crucial role in carcinogenesis as they can regulate cellular growth and differentiation by reprogramming the transcriptome [156, 157]. In recent years, their pivotal role in CSCs progression and stemness maintenance has been studied extensively and it is well-mentioned that they are key regulators of stemness properties and stem cell plasticity [158, 159]. For example, the lncRNA MALAT1 and THOR maintain gastric cancer stemness by regulating SOX2 expression post-transcriptionally. This lncRNA binds to the SOX2 mRNA and strengthens its stability and expression [160, 161]. Similarly, lncRNA-HAND2-AS1 has been found to increase stemness of HCC and non-small cell lung cancer (NSCLC) through activating BPM signaling and suppressing TGF-β1 respectively [162, 163]. Correspondingly, lncGATA6 perpetuates intestinal cancer stemness and stimulates tumorigenesis [164]. In AML, the enhanced expression of HOTAIR in LSCs led to the inhibition of p15, which is a tumor suppressor gene. Thus, lncRNA could serve as a potential therapeutic marker as its knockdown could increase the survival time of AML patients [165]. Furthermore, some oncogenic alterations could also affect the transcription of lncRNAs which are necessary for CSCs stemness maintenance and chemoresistance [166].

Correspondingly, elevated level of miRNAs has been found in different malignancies to promote stemness features. In HCC, the epigenetically altered miR-494 has been found to be linked with stem cell phenotype and sorafenib resistance [167]. Another study shows that miR-217 enhanced hepatocellular carcinoma stem cells phenotype via targeting *DkK1* gene expression [157]. While in cutaneous squamous cell carcinoma, miR-142-5p produced CSCs by inhibiting *PTEN* [168]. Besides regulating stemness-associated factors, these non-coding RNAs also participate in the regulation of the process of EMT [159]. BCSCs, the most studied CSC in solid tumors, prevail in two dynamic states i.e. mesenchymal-like (CD24$^-$CD44$^+$) and epithelial-like (ALDH$^+$). This interconversion aids in the invasion and dissemination of breast tumors at distinct sites. Studies also illustrate that different miRNAs, in particu-

lar, miR-9, miR-100, miR-221 and miR-155 give raise to EMT state, whereas, miR-93, miR-200 and miR-205 are associated with the induction of mesenchymal-to-epithelial state [159, 169].

The lncRNA/miRNA regulatory network is highly complex as lncRNA also harbors the ability to silence miRNA. In colorectal cancer, lnc34a epigenetically masks miR-34a to induce asymmetric division of CSCs [170]. On the other hand, LINC00337 in cervical cancer acts like a competing endogenous RNA (ceRNA) of miR-145, where it positively regulates KLF5 translation by inhibiting miR-145 [171]. These noncoding RNAs are not always oncogenic as their tumor suppressor role has also been reported. In recent years, Chen and companions have discovered a novel lncRNA (lnc-LBCS) that inhibits bladder cancer tumorigenesis and chemoresistance by inhibiting SOX2 protein expression [172]. Whereas, miR-15b suppresses TRIM14 to drive cisplatin sensitivity and inhibit CSCs in oral tongue squamous cell carcinoma [173]. Furthermore, depletion of HOTAIRM1 upgraded self-renewal of CSCs and tumor aggressiveness in colorectal cancer and uveal melanoma [174]. In the same fashion, miRNA, let-7g-5p inhibited GBSCs phenotypes by reducing EMT via downregulating VSIG4 [175].

Epitranscriptomics

With the advancement of sequence databases and transcriptome-wide mapping, various post-transcriptional modifications have been identified in both coding and non-coding RNA molecules. At first, these modifications were presumed to be limited to only tRNA and rRNA molecules. However, further investigations revealed that these changes also exist in mRNA molecules. Thus, an emerging group of RNA variations led to the foundation of epitranscriptomics in 2012 [176]. Although previously (epi) genetic alterations, transcription factors and cell signaling pathways were thought to be responsible for stem cells regulation and fate decision, increasing data suggest that RNA-modifications also cause strong dramatic and dynamic changes during cellular reprogramming or lineage decision [177, 178]. Hence, epitranscriptomics has emerged as an additional regulatory layer in CSCs stemness maintenance and lineage commitment. Therefore, recent studies are focusing on the role of these RNA modifications in CSCs' self-renewal differ-

entiation and proliferation. To date, more than 170 different RNA alterations have been reported [179]. Increasing evidence suggests that among these distinct modifications, the two RNA-based modifications: N^6-methyladenosine (m^6A) and adenosine-to-inosine (A-to-I) play a significant role in CSCs regulation and maintenance [176, 180].

N^6-methyladenosine modifications (m^6A): N^6-methyladenosine is one of the most frequent and prevalent RNA modifications that has been observed in almost all types of RNAs (mRNA, rRNA, tRNA, lncRNA, miRNA and cirRNA) [176, 181]. This process of RNA editing involves methylation in the N^6-position of adenosine that is mediated by methyltransferases (METTL3/14/16, CBLL1, ZC3H3, WTAP, RBM15/15B, VIRMA and KIAA1429), erased by demethylases (ALKBH5 and FTO) and identified by the reader proteins (IGF2BP1/2/3, YTHDF1/2/3, YTHDC1/2 and hnRNPA2B1) [182]. Accumulating evidence suggests that m^6A acts as a molecular switch to control embryonic development [183, 184]. In a transcriptome-wide analysis, m^6A was found to induce abundant alterations in the core pluripotency factors i.e. SOX2, OCT4, NANOG, and KLF4 [183]. Moreover, METTL3 depleted mice exhibited increased half-life of these key pluripotency genes, which resulted in embryonic lethality [183]. The m^6A modifications also control the dedifferentiation of cancer cells. For instance, depletion of m^6A methylation led to NANOG translation that caused the reversion of cancer cells to their primitive pluripotent state [185]. Similarly, another group observed increased expression of FTO in leukemia that reduced m^6A methylation of RARA and ABS2, thus promoting AML growth and proliferation [186]. FTO-mediated demethylation has also been observed to regulate self-renewal of GBSCs as its inhibition by MA2 impaired GBSCs proliferation and self-renewal [187]. In the same manner, ALKBH5 was found to be highly expressed in GBSCs and its depletion disturbed the proliferation of patient-derived GBSCs by inhibiting FOXM1 expression [188]. Surprisingly, a lncRNA antisense to FOXM1 promoted GBSCs tumorigenesis by accelerating FOXM1 and ALKBH5 transcripts interaction [188]. On the contrary, aberrant m^6A alterations promote BCSCs generation [189]. Also, in AML, increased METTL3 activity has been observed that increased translation of

c-MYC and BCL2 [190]. Similarly, elevated level of METTL14 has been found in AML cells that lead to the upregulation of MYB and MYC mRNA to regulate LSCs [191]. Visvanathan and coauthors reported that MELTT3-mediated methylation also promotes glioma cancer stem cells dedifferentiation [192]. They also showed that MELTT3 targets SOX2 to sensitize glioma cancer stem cells to γ-radiation [192]. Finally, in colorectal cancer, increased expression level of m6A reader YTHDF1 has been found to assist self-renewal and colonosphere formation via increasing Wnt/β-catenin pathway activity [193]. Overall, depending upon the type of cancer both m6A-mediated methylation and demethylation regulate tumorigenesis by modulating CSCs stemness.

Adenosine to Inosine (A-to-I) editing: A-to-I modification is another important post-transcriptional modification observed in eukaryotes. In this process, hydrolytic deamination of adenosine takes place, which is catalyzed by ADAR-RNA editases: ADAR1, ADAR2 and ADAR3 [194]. During mRNA translation this newly generated inosine base is read as guanosine by the ribosome, thus causing A-to-G transcriptome diversity if occurred in the protein-coding region [195]. A-to-I editing plays an important role in embryonic development and growth. Hartner and his group showed that ADAR1 depletion in mice caused hematopoietic deficits that led to embryonic lethality [196]. Similarly, ADAR2 deficient mice were born and developed normally but died after three weeks of birth [197]. Keeping in view the importance of A-to-I modification in growth and development, it is not surprising that it also participates in CSCs regulation. Notably, A-to-I modifications have been found to regulate stemness features in hematopoietic malignancies. According to Lazzari and coworkers, around 30-50% of multiple myeloma patients show ADAR1 amplification that favors poor prognosis [198]. In another study, ectopic expression of ADAR1 in LSCs led to myeloid progenitor cells expansion and enhanced expression of GSK-3β that promotes LSCs self-renewal by activating β-catenin [199]. Furthermore, ADAR1 knockdown inhibited *in vivo* engraftment capability of multiple myeloma via attenuating GLI1 activity [198]. ALDAR1 also caused mutation in GLI1 by editing its exon 12, which caused inhibition of HH pathway negative regulation [198]. In a tremendous study,

Jiang and collaborators have identified that ADAR1 downregulation may serve as an efficient way to eradicate dormant imatinib-resistant LSCs. According to this study, ADAR1 controls the cell cycle of progenitor cells by inducing A-to-I editing, which led to increase MDM2 expression and p53 inactivation [200].

Besides mRNA, ADAR1-mediated A-to-I editing has also been observed to regulate the cell cycle by deregulating the formation of tumor suppressor miRNAs, leading to accelerate the self-renewal of CSCs. For example, wild-type ALDAR1 was found to inhibit tumor suppressor miR-277 expression, which targets STAT5 to restore imatinib sensitivity in CML [200]. Similarly, A-to-I editing caused inhibition of cell cycle proliferation-associated miR-411 [200]. Lastly, the expression of miR-26a-5p (target of master pluripotency factor MYC) was also repressed by ADAR1 mediated inosine editing [200]. These findings suggest that ADAR1 mediated RNA editing is pivotal for LSCs self-renewal and stemness maintenance. Hence, further studies should be conducted on the elucidation of the role of RNA-based alterations in the regulation of CSCs, which may serve as a novel biomarker for CSCs' elimination.

Extrinsic factors

<u>*The cancer microenvironment*</u>

Cancer cells remain ingrained in a complex microenvironment that is usually comprised of various stromal cells, immune cells, cancer secretory factors, hypoxic regions and extracellular matrix [201]. The cancer microenvironment contributes significantly to the modulation of CSCs phenotype. In light of a recent study, it can be stated that external cues from the TME induced phenotypic drift in CSCs. In this study, temozolomide treatment under hypoxic conditions transformed glioma stem cells (GSCs) and their progeny into various cell types, by modifying their surface marks [202]. A contemporaneous study ratifies this view by stating that stimulation of luminal-A breast cancer cells with a combination of tumor microenvironmental factors including TNF-α, epidermal growth factor and estrogen, promoted intratumor heterogeneity [203]. Such a microenvironmental network enriched breast cancer cells with chemotherapy-resistant and highly metastatic CSCs with CD44/CD24L phenotype

that exhibited high plasticity [203]. It is well-known that an ample number of mesenchymal stem cells are recruited to the stem cell niche. Exposure of MSCs to cancer cell-secreted soluble factors transformed them to chemotherapy-resistant cancer stem-like cells. Further stimulation of these cells with TGF-β enhanced their mesenchymal phenotype and invasion potency [204]. Cancer-associated fibroblasts, a major constituent of tumor stroma, were found to regulate liver cancer-initiating cells via releasing HGF [201]. Accordingly, interferon regulatory factor-7 produced an inflammatory microenvironment to induce angiogenesis and heterogeneity in glioma. It capacitated glioma cells to acquire GSCs state by releasing interleukin-6 [205]. Altogether, CSCs' fundamental traits and heterogeneity are greatly influenced by different extrinsic factors elicited from the cancer microenvironment.

Cancer stem cell's heterogeneity and cancer metastasis

Cancer dissemination is considered a major obstacle in oncology. Albeit, various attempts have been made to mitigate and eradicate primary tumors completely, the successful progression of metastatic lesions makes cancer a leading cause of mortality. Accumulative evidence represents that cancer stem cells, the key drivers of cancer metastasis, are genetically and phenotypically diverse in metastatic sites as compared to primary tumors. While studying breast cancer metastasis initiating cells, Lawson and his group unveiled a hierarchical model for metastasis. They found that metastasis cells obtained from low and high burden tissues were heterogeneous. As low-burden metastatic tissues expressed elevated level of stemness, dormancy, EMT and anti-apoptosis genes, while metastatic cells within high burden metastasis tissue were more heterogeneous with increased proliferation and differentiation capacity [206]. The expression level of stemness-associated genes was also found to be diverse in primary and metastatic sites. For instance, LGR5, a well-known marker for CCSCs, expression was remarkably greater at tumor-infiltrating front in contrast to tumor expanding front [207]. Similarly, more CSCs were found in the highest metastatic breast cancer model than in the lowest metastatic model [206].

Circulating tumor cells (CTCs) are associated with enhanced metastasis risk as in the presence of a favorable microenvironment CSCs in CTCs enable them to initiate metastasis [208, 209]. A recent study shows that breast cancer CTCs express multiple CSCs marks [208]. Lyberopoulou and coworkers found genetic discordance between CTCs and primary tumors in 5-10% of cases [210]. The existence of a variant isoform of a particular subset of CSCs is also plausible that participates in dissemination. Identifying BCSCs heterogeneity Hu and colleagues reported that CD44v, a variant isoform of CD24$^-$/CD44$^+$/CSCs, was responsible for lung cancer metastasis [211]. Moreover, the expression of CD44v6 was higher in metastatic lesions compared to the initial tumor [80]. Recent studies present a dynamic picture of CSCs in metastasis settings [212, 213]. In the journey of establishing metastasis, colorectal CSCs first lose the LGR5 expression mark hence, LGR5$^-$ cells migrate to the metastasis site and then convert back to the LGR5$^+$ state for effective metastatic growth [213]. These observations illustrate that CSCs plasticity and heterogeneity play a significant role in cancer dissemination as it enables metastatic cells to adopt the new microenvironment in the secondary site (**Figure 3**).

### Cancer stem cell's heterogeneity and therapy resistance

Although various modalities have been proposed for the effective treatment of cancer, chemotherapy has remained one of the most extensively used methods for the treatment of many types of cancers [214]. Lately, remarkable chemotherapeutic agents have been designed to eliminate primary tumor lesions and their residues which remain left after surgery or radiotherapy. However, heterogeneity in cancer cells renders them resistant to the effects of chemotherapeutic agents [215]. Hence, chemotherapy resistance is a pivotal obstacle in the meaningful outcome of cancer treatment. It is increasingly appreciated that CSCs are more refractory to anticancer therapies as compared to their differentiated counterparts [216]. Therefore, enrichment of CSCs [215] or elevated level of stemness-associated factors like SOX2, OCT4 and NESTIN have been observed in cancer cells upon chemotherapies [217]. Besides, another group of researchers repre-
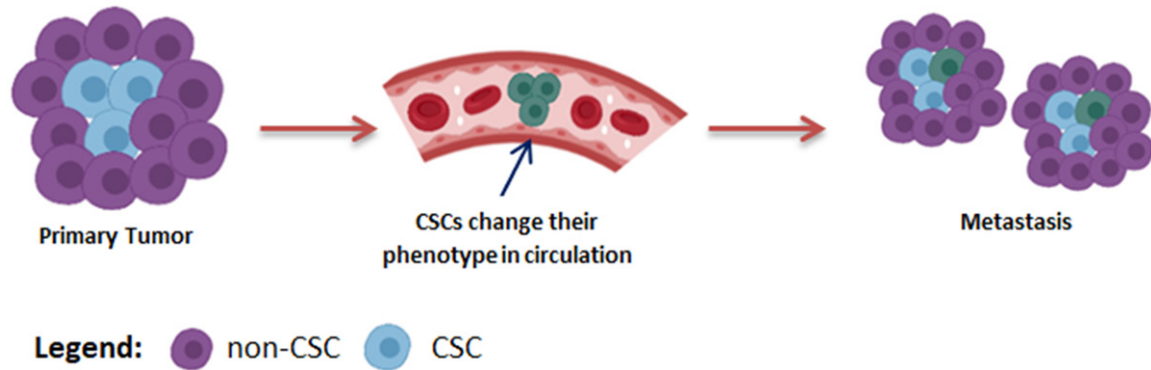
Figure 3. CSCs heterogeneity and plasticity favour metastasis. Cancer stem cells change their phenotype to assist metastasis at secondary site, where they either regain primary tumor characteristics or remain changed.
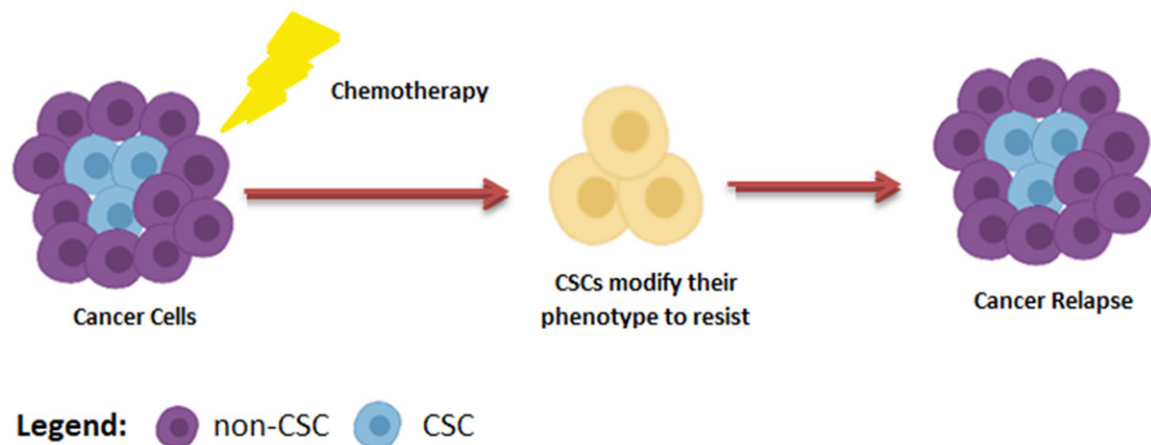
Figure 4. CSCs Heterogeneity favour therapy resistance. Upon chemotherapy CSCs modify their phenotype to resist therapy.

sented that rather than enrichment of CSCs, phenotypic plasticity drives drug resistance [218]. From the CSCs viewpoint, different mechanisms exist that allow therapy to establish tumor heterogeneity. At first, chemotherapy favors the selection of CSCs to change intra-tumor heterogeneity as CSCs harbor the ability to instigate cancerous cells in diverse heterogeneous lineages upon exposure to chemotherapy [215]. Similarly, some cytotoxic drugs like temozolomide can also induce heterogeneity in CSCs which ultimately aid in therapy resistance [219].

Phenotypic plasticity is another mechanism by which therapy can induce heterogeneity in tumor mass. For instance, by utilizing the EMT phenomenon, CSCs expeditiously shifts from the chemotherapy-sensitive (epithelial) phenotype to the chemotherapy-resistant (mesenchy-

mal) phenotype [220]. Recent literature shows that cancerous cells also exploit epigenetic modifications as a protective mechanism to shift phenotypes and escape chemotherapy. As KDM5B epigenetically converts melanoma propagating cells from CD34^{+} state to CD34^{-} state to escape vemurafenib [221]. In an elegant study, sherma and coworkers carried out a detailed analysis of patient-derived oral squamous cell carcinomas primary cells evolutionary mechanism to cisplatin resistance. They revealed that pre-existing heterogeneity favors the selection of drug-resistant CSCs clones, while within homogenous cancer population epigenetic reprogramming caused transdifferentiation of CSCs into the drug-resistant state [222]. Therefore, it can be stated that CSCs heterogeneity and plasticity is a major hurdle in the effective eradication of the tumor (**Figure 4**). Hence, new anticancer therapies should be

designed to kill CSCs in parallel of targeting bulk tumor cells.

Targeting cancer stem cells

Considering the importance of CSCs in developing tumor heterogeneity, metastasis and therapy resistance, it is important to design novel strategies against them. Undoubtedly, the eradication of CSCs will effectively improve the prognosis and survival rate of cancer patients. In recent decades, several new modalities have been proposed to eliminate CSCs present in the bulk tumor mass. These include disrupting regulatory signaling pathways, targeting CSCs surface markers, disturbing TME and immunotherapy.

Targeting CSCs associated signaling pathways

As mentioned in the previous section, cell signaling pathways play a vital role in the formation and regulation of CSCs. Mainly Wnt, Notch and HH pathways are found to be altered in CSCs as these are the key regulators for the maintenance and survival of these cancer propagating cells. Therefore, these pathways have gained increased attention from researchers to cure cancer.

Targeting notch pathway

The Notch pathway has been found abnormally expressed in many cancers, like leukemia, glioblastoma and lung, breast, colon and pancreatic cancer [223]. Its abnormal functioning supports different aspects of cancer biology i.e. CSCs differentiation, immune evasion, metastasis and angiogenesis [224]. Currently, the three main approaches used to suppress Notch signaling include gamma-secretase inhibitors (GSI), Notch receptors inhibitors, and their combination with other therapies. GSI, the first Notch inhibitor to enter clinical trials, showed potent antitumor activity in different malignancies. For instance, MK-0752 exhibited targeted inhibition of pediatric brain tumors in phase I clinical trials [225]. PF-03084014, an oral GSI, showed anti-neoplastic activity in desmoid tumors in both phase I and II clinical studies [226]. Similarly, MRK-003 expressed anti-CSCs activity in breast cancer [227]. GSI also acts as an adjuvant to chemotherapeutic drugs to eliminate cancer. For example, the combination of PF-03084014 with docetaxel enhanced the chemosensitivity of PrCSCs to docetaxe [228]. Likewise, the combination of DAPT with cisplatin repressed growth and metastasis of lung-resistant osteosarcoma cells [229]. Similar to GSI, DLL4 inhibition also showed promising results to suppress Notch pathway activation. Demcizumab, a humanized monoclonal antibody against DLL4, remarkably decreased tumor growth and CSCs markers in non-small cell lung, breast, pancreatic, ovarian and colon cancers, when used in combination with chemotherapy [230]. It also suppressed CSCs formation and tumor growth in advanced malignancies [230]. ABL001 is another therapeutic antibody, that was found to inhibit GCSCs population, metastasis and invasion either alone or in the combination with irinotecan [231]. Xie and coauthors have identified that increased expression of DLL4 is linked with sunitinib resistance in metastatic renal cell carcinoma. Hence, inhibiting DLL4 and VEGF may serve as an efficient strategy to cure tumors of interest [232].

Targeting hedgehog pathway

Targeting the HH pathway is another strategy to inhibit CSCs proliferation. It also participates in embryonic development and its aberrant activation has been observed in different malignancies [233]. The mechanism of HH pathway-mediated transcription regulation mainly depends upon SMO proteins that cause activation and nuclear transfer of respective transcription factors. At present, FDA has approved three different SMO antagonists to cure basal cell carcinoma (BCC) and AML. These include vismodegib, sonidegib and glasdegib [27].

Vismodegib was the first cyclopamine competitive antagonist, approved in 2021 for metastatic and advanced BCC. In the overall survival analysis of BCC patients, it was found that it can increase the median survival duration to 2.8 years, which was computed as only 2 years with standard treatments. Afterward, exploring the efficacy of vismodegib in medulloblastoma, it was found that it can improve progression-free survival (PFS) of SHH-subtype medulloblastoma patients than the non-SHH subtype cases. Furthermore, vismodegib also showed anti-neoplastic activity in the colon and oral squamous cell carcinoma [234, 235]. After vismodegib, FDA approved sonidegib for the treat-

ment of surgery and radiation therapy-resistant BCC patients. In a phase I study of extensive grade small cell lung cancer patients, sonidegib was found effective to sustain PFS when used in combination with etoposide and cisplatinin SOX2 amplified patients [236]. Subsequently, in a phase II trial, it was found to cure 50% of recurrent medulloblastoma patients [237]. Sonidegib has also been found to affect CSCs properties and decreases carcinogenesis in glioblastoma, prostate cancer and renal cancer [238-240].

In 2018, FDA approved glasdegib for the treatment of newly diagnosed AML patients. In phase I trial, it displayed significant results for the treatment of partial hematologic malignancies in Japanese patients [241]. Moreover, in the phase II study, glasdegib in combination with cytarabine and daunorubicin showed promising results to cure AML and high-risk myelodysplastic syndromes [242]. Similarly, the combination of glasdegib and low dose cytarabine was found effective for AML patients which were not appropriate for intensive chemotherapy [243]. Besides these, some other SMO antagonists like taladegib and saridegib have been tested to treat advanced solid tumors and medulloblastoma respectively [244, 245].

Targeting Wnt pathway

Activation of the Wnt signaling pathway is also implicated in a variety of cancers [27]. At present, various inhibitors of this pathway are in clinical testing, while most of the anti-Want drugs are in preclinical trials. Ipafricept is a high-quality recombinant protein, that inhibits the Want pathway by targeting FZD8 [246]. Correspondingly, vantictumab inhibits the Wnt signaling by binding with the FZD family receptors. It also reduced the number of CSCs and the size of tumor growth in patient-derived xenograft models of different tumors. Besides, it also exhibited synergy with various chemotherapeutic drugs [247]. Cirmtuzumabis another antibody that antagonized Wnt signaling to suppress stemness signatures in CML patients by targeting the ROR1 receptor [247]. PRI-724 and CWP-2322 are β-catenin inhibitors effective for advanced myeloid malignancies and AML respectively [248]. Recent literature also highlights the effectiveness of using Wnt inhibitors prior to standard-of-care chemotherapeutic medications [249]. Moreover, the com-

bination of C4 and nilotinib has been found efficacious to inhibit Wnt pathways in CML stem cells [250]. Similarly, the triple combinations of ipafricept, NCT02069145 and sorafenib for metastatic pancreatic cancer, Wnt-974, cetuximab and encorafenib for metastatic colorectal cancer and vantictumab, gemcitabine and nab-paclitaxel for HCC are also investigated.

Targeting CSCs microenvironment

The TME serves as an ideal place for maintaining differentiation and tumorigenicity of CSCs. in recent decades, different therapeutic agents have been designed to inhibit signals from the cancer microenvironment, that aid generation and propagation of CSCs. VEGF is an important immunological regulator in the TME [251]. In hypoxic conditions, it causes induction of immunosuppressive cells and vascular endothelial cells to support tumorigenesis [251]. Pristimerin, a natural compound isolated from Hippocrateaceae and Celastraceae, was found to reduce prostate cancer bone metastasis by inhibiting VEGF-mediated vasculogenesis in endothelial progenitor cells [252]. CXCL12 is another important constituent of the TME. It works in association with the CXCR4 receptor to assist cancerous cells to communicate with their microenvironment. Plerixafor is a potent therapeutic drug that targets CXCL12/CXCR4 axis in hematological malignances. LY2510924 also antagonizes CXCR4 in patients with advanced cancer. In addition, the combination of VEGF and CXCR4 antagonists (mcr84 and POL5551 respectively) has been found effective to increase the survival time of glioblastoma patients by targeting the perivascular niche [253]. Similarly, the combination of plerixafor and high-dose etoposide and cytarabine has shown successful results to cure pediatric resistant or relapsed acute leukemia and myelodysplastic syndrome [254]. Cancer-associated fibroblasts are a heterogeneous group of activated fibroblasts that regulate tumorigenesis in a variety of ways [255]. Some of their subsets have been recognized to regulate CSCs by promoting their generation and propagation [255]. Therefore, directly disrupting the results in improved clinical outcomes. To date, different antagonists have been designed to target surface markers present on their surface. For example, targeting FAP by sibrotuzumab, S1004A by 5C3 and ADC by TEM8 cured different tumors [256, 257]. TGF-β is one of the

major secretions of CAFs, that regulate cancer stemness and metastasis. Inhibition of TGF-β signaling by LY364947 based nanotherapy was found to be effective to clear BCSCs [258]. In the cancer microenvironment, M2 featured tumor-associated macrophages are found abundantly. Overexpression of the SRC gene in M2-like tumor-associated macrophages caused cisplatin resistance [259]. A recent study revealed that dasatinib treatment caused the elimination of stemness markers and cisplatin-resistant NSCLC [259].

Targeting CSCs-associated surface markers

Targeting CSCs surface markers has emerged as a novel strategy to eliminate cancer. Several monoclonal antibodies have been developed against CSCs biomarkers. Bivatuzumab is an effective monoclonal antibody against CD44v6, which is expressed in many cancers such as melanoma, breast, ovarian, lung and colon cancer [260, 261]. Talacotuzumab has been found efficacious to eliminate CD16 and CD123 [262]. Accordingly, adecatumumab can cure hormone-resistant prostate cancer by targeting EpCAM. Recently, a group of researchers has developed a humanized anti-CD271 antibody for targeting CSCs in melanoma and hypopharyngeal cancer [263]. On the contrary, another group has discovered ROR1 specific antibody to inhibit chemotherapy-refractory BSCs [38]. Although significant progress has been made to design antibodies against CSCs surface markers, their plastic nature enables them to evolve in distinct subtypes upon treatment. Therefore, it is important to determine CSCs' phenotype prior to treatment. Moreover, antibodies in combination with appropriate chemotherapeutic drugs should be used to obtain an optimal therapeutic outcome.

Immunotherapy

Paul Ehrlich, in his pioneer study, convinced the idea that a healthy immune system can fight cancer [27]. Based on this idea, cancer immunotherapy has been emerged as a novel approach to suppress cancer. Recent advances in cancer immunotherapies showed that this approach eradicates CSCs effectively. Different immunotherapies to target CSCs include dendritic cell-based vaccine, adoptive T-cells therapy, oncolytic virotherapy, immune checkpoint inhibitors and combined therapies [264]. Among these, anti-CTLA-4, PD-1 and PD-L1 antibodies have provided significant clinical outcomes in patients with advanced malignancies [265, 266]. However, in the majority of cases, the use of single antibodies has remained limited due to the poor treatment response. Thus, combinatorial approaches provide a more remarkable response and increased recovery rate. For example, in the mouse melanoma model, the simultaneous use of anti-PD-L1 and anti-CTLA-4 antibodies along with anti-CSCs dendritic vaccine promoted tumor eradication by eliminating ALDH^H [267]. Likewise, oncolytic herpes virus expressing IL-4 along with anti-PD-1 and anti-CTL-4 antibodies cured glioblastoma in mouse models [268]. In addition, PD-1 blockade has been reported to increase the anticancer activity of bladder cancer stem cells vaccine [269].

Conclusion

Cancer is a complex illness that is hard to cure due to its heterogeneity. Recent studies regard CSCs as the main cause of cancer heterogeneity. They make cancerous cells resistant to the therapies and escalate the processes of cancer dissemination, recurrence and therapy resistance. In light of the discussed data, it is evident that both cell intrinsic and extrinsic factors promote cancer aggressiveness by inducing CSCs heterogeneity. Hence, CSCs along with the bulk cancer cells must be targeted to achieve a fruitful outcome of cancer therapy. However, the targeted removal of preexisting CSCs is inadequate as cellular reprogramming induces the generation of de novo CSCs from the differentiated non-cancer cells with divergent characteristics. Therefore, it is needed to design new strategies to target stem cell heterogeneity and plasticity and associated factors that establish and maintain this diversification.

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Cancer stem cells: a major culprit of intra-tumor heterogeneity

Disclosure of conflict of interest

None.

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